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EXHIBIT 3

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Correspondence and requests for materials should be addressed to K.N. (knomurscb@mbox.nc.kyushu-u.ac.jp) or K.S. (k-sugar@kobepharma-u.ac.jp). Sequences of the longer ChSy and the shorter ChSy have been deposited in the DNA Data Bank of Japan under accession numbers AB088397 and AB088398, respectively.

Wnt proteins are lipid-modified and can act as stem cell growth factors

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Wnt signalling is involved in numerous events in animal development[1], including the proliferation of stem cells[2] and the specification of the neural crest[3]. Wnt proteins are potentially important reagents in expanding specific cell types, but in contrast to other developmental signalling molecules such as hedgehog proteins and the bone morphogenetic proteins, Wnt proteins have never been isolated in an active form. Although Wnt proteins are secreted from cells[4–7], secretion is usually inefficient[8] and previous attempts to characterize Wnt proteins have been hampered by their high degree of insolubility. Here we have isolated active Wnt molecules, including the product of the mouse *Wnt3a* gene. By mass spectrometry, we found the proteins to be palmitoylated on a conserved cysteine. Enzymatic removal of the palmitate or site-directed and natural mutations of the modified cysteine result in loss of activity, and indicate that the lipid is important for signalling. The purified Wnt3a protein induces self-renewal of haematopoietic stem cells, signifying its potential use in tissue engineering.

We expressed several Wnt genes, including *Wnt3a* (ref. 9), in a variety of cell lines and generated antibodies to monitor Wnt protein secretion into the medium. For purification purposes, we selected clones of cells secreting the highest amounts of protein (200 ng ml^{-1} for Wnt3a from mouse L (L-M[TK$^-$]) cells). We tested the activity of Wnt3a by assaying its ability to stabilize cytosolic β-catenin, a known target and signal transduction component of Wnt signalling[10]. Mouse L cells accumulate high levels of β-catenin protein after a 2-h incubation with Wnt3a-conditioned medium (Fig. 1b, top panel; see also ref. 11).

Initial characterization of secreted Wnt3a indicated that it is hydrophobic (see below), therefore we designed a purification protocol that starts with chromatography on blue (Cibacron blue 3GA) Sepharose in the presence of the detergent CHAPS. Under these conditions, Wnt3a binds with high selectivity to the resin and can be eluted in a relatively pure form by increasing ionic strength (Fig. 1a and Table 1). Approximately 60% of added Wnt3a is recovered in this step with nearly 2,500-fold enrichment. We then separated Wnt-containing fractions by size exclusion chromatography on a Superdex 200 column, and finally by cation exchange on heparin (Table 1). These steps yielded fractions of Wnt3a that were greater than 95% pure as assessed by Coomassie staining (Fig. 1a). Through size exclusion chromatography, we determined that active Wnt3a is monomeric (not shown).

We have applied successfully similar purification methods to a variety of other Wnt proteins, including *Drosophila* Wnt8 (Fig. 1a), mouse Wnt5a and *Drosophila* Wingless (not shown).

Throughout the purification, we measured the ability of Wnt3a to stabilize β-catenin in L cells. The final purified product exhibited no loss in activity compared to the original starting material (Fig. 1b). The purified Wnt3a protein retains the range of activities expected for a Wnt protein. For example, we tested the effect of Wnt3a protein on *Xenopus* animal cap explants and found that two known target genes, *siamois* and *Xnr3* (refs 12, 13), are induced by Wnt3a (Fig. 1c). As a further assay for Wnt activity, we used C57MG cells, a line derived from the mouse mammary gland that can be morphologically transformed by *Wnt* gene expression[8]. Purified Wnt3a promotes the morphological transformation of these cells (Fig. 1d) similar to that of *Wnt* gene transfection. Furthermore, the protein can induce expression of known transcriptional Wnt targets including *MSX1*, cyclin D1 and *MYC* in human teratocarcinoma cells (data not shown).

All purification steps required the presence of detergent to maintain solubility and activity, suggesting that Wnt proteins are hydrophobic. We used the two-phase separation property of the detergent Triton X-114 (ref. 14) to test this. Most Wnt3a partitioned to the detergent phase (Fig. 2a), a behaviour characteristic of highly hydrophobic proteins such as integral membrane proteins. As the primary amino acid sequence of secreted Wnt does not contain long stretches of hydrophobic residues, we used metabolic labelling to test whether Wnt is post-translationally modified by lipid attachment. We found that the protein is labelled with tritiated palmitate (Fig. 2b).

Evidence for the functional importance of the lipid modification

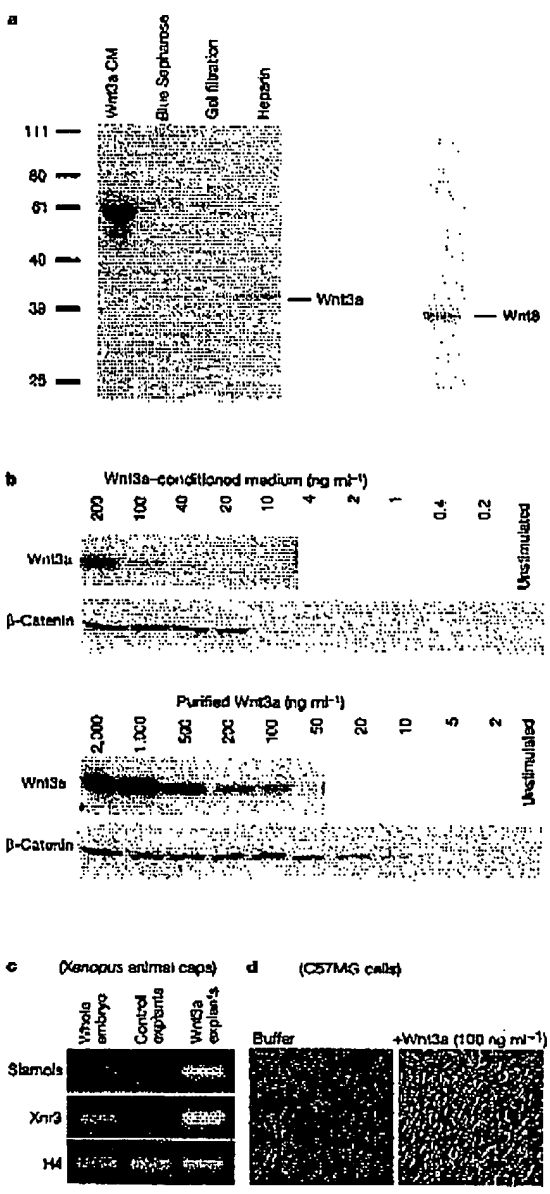


Figure 1 Wnt3a and *Drosophila* Wnt8 purification. a, Coomassie staining of an SDS polyacrylamide gel containing fractions from all steps of the purification reveals the enrichment of the Wnt3a protein. Also shown is the final *Drosophila* Wnt8 fraction, purified using the same protocol. Size markers are in kilodaltons. b, Wnt3a stabilizes the β-catenin protein. Wnt3a-conditioned medium (200 ng ml^{-1}) and purified Wnt3a (100 µg ml^{-1}) was diluted as indicated in medium containing 10% FBS and detected by western blot. L cells were stimulated for 2 h. c, Wnt3a induces expression of *siamois* and *Xnr3* in animal cap explants of *Xenopus* embryos. Animal cap explants were incubated with 100 ng ml^{-1} Wnt3a and analysed by polymerase chain reaction with reverse transcription for expression of the direct targets *Xnr3* and *siamois*. d, Wnt3a induces the morphological transformation of C57MG cells. C57MG cells were treated with or without 100 ng ml^{-1} Wnt3a for 2 days in serum-containing medium and then an additional 2 days in serum-free medium.

came from treatment of Wnt3a with acyl-protein thioesterase-1 (APT-1), an enzyme that removes palmitate from G proteins and other thioacyl protein substrates[15]. This treatment shifts Wnt3a to the water phase in the Triton X-114 phase separation experiment (Fig. 2c), suggesting that APT-1 removes a thioester-linked lipid, such as palmitate. APT-1 also blocks the ability of Wnt3a to stabilize β-catenin (Fig. 2c).

To map the lipid attachment site on the Wnt polypeptide we subjected proteolytic peptide fragments of both Wnt3a and *Drosophila* Wnt8 to liquid chromatography tandem mass spectrometry, which identifies molecular masses of the ionized peptides and obtains primary amino acid sequence information through collision-induced fragmentation. In both proteins we identified ions whose masses were consistent with the addition of 238 daltons (the mass of palmitate is 256 daltons accounting for the loss of water in the formation of a thioester linkage) and which produced fragmentation data consistent with a peptide containing a conserved cysteine modified by palmitate (C77 in Wnt3a and C51 in *Drosophila* Wnt8; underlined in Fig. 2d). This cysteine is absolutely conserved among all Wnt family members (bold in Fig. 2d); it is the most amino-terminally conserved cysteine of the Wnt family (http://www.stanford.edu/~rnusse/genealigns/manywnts.html).

To test for the requirement of C77 in cell culture, we mutated it to alanine in Wnt3a and expressed the mutant protein (Wnt3a(C77A), Fig. 2c) in 293 and in L cells. The mutant Wnt3a protein was secreted at levels similar to that of the wild-type protein. This indicated that the mutation, unlike many other cysteine mutations in Wnt proteins[16], does not interfere with the folding of the protein. However, when the Wnt3a(C77A) protein was subjected to the Triton X-114 phase separation test, it partitioned in the water phase, indicating that it had lost its hydrophobic character (Fig. 2a). In a β-catenin assay on L cells, Wnt3a(C77A) was not active over a range of concentrations tested (Fig. 2e, left). In a transfection assay on 293 cells however, there was a noticeable increase in the intracellular levels of β-catenin, demonstrating that the Wnt3a(C77A) mutant retains some activity when expressed at high levels in an autocrine manner (Fig. 2e, right).

Notably, a natural loss-of-function allele of the *Caenorhabditis elegans egl-20* gene (*egl-20(N585)*; ref. 17) contains a serine replacing the cysteine corresponding to C77 (Fig. 2d). Moreover, in a survey of *wingless* (*wg*) alleles in *Drosophila*, we found that the *wg*^{521} allele[18] contains a tyrosine instead of that same cysteine (Fig. 2d). Thus, our data are consistent with the lipid modification being important for Wnt signalling activity. At the moment, we cannot exclude the possibility that Wnt proteins carry other modifications beyond palmitoylation and *N*-linked glycosylation; nor can we rule out that different forms of Wnt proteins (that is, cell-bound) are palmitoylated at other sites.

Next we investigated whether Wnt3a can be used as a reagent to control cell fate in a well-characterized stem cell system, through application of the isolated protein to purified haematopoietic stem cells (HSCs)[19]. Single HSCs responded well to the Wnt3a protein in the presence of limiting doses of steel factor (SLF). Over a period of 7 days, the frequency of cells proliferating was 5.8-fold greater compared with control conditions (Fig. 3a, b). Most of the cells (82%) were undifferentiated, as they did not express markers for differentiated lineages. Thirty per cent of the lineage-negative cells expressed c-Kit and Sca-1, consistent with an HSC phenotype, whereas 64% were at the stage of myeloid progenitors (c-Kit^{+}, Sca-1^{-}; Fig. 3c, d). In contrast, incubation of HSCs with unfractionated Wnt3a-conditioned medium, in which Wnt3a itself is present at a similar concentration, resulted in a significant fraction (86%) of the cells expressing markers specific for differentiated lineages (Fig. 3c). This suggests that conditioned medium contains factors not present in purified Wnt3a that promote differentiation, underscoring the importance of having purified Wnt proteins available for the purpose of maintaining the self-renewing fate of HSCs.

Table 1 Purification table

	Volume (ml)	Protein concentration	Total protein (mg)	Wnt3a concentration	Wnt3a (μg)
Wnt3a CM	2,000	4.46*	8,920	200†	400
Blue Sepharose	80	36.0†	2.16	4‡	240
Gel filtration	36	17.1†	0.615	5†	180
Heparin cation exchange	1.15	104†	0.120	100†	116

The concentration of Wnt3a protein in the conditioned medium (CM) was determined by comparing its signal intensity on a Wnt3a immunoblot to that of a serial dilution of a known amount of purified Wnt3a protein.
* Concentration in mg ml^{-1}.
† Concentration in μg ml^{-1}.
‡ Concentration in ng ml^{-1}.

To determine whether the cells that proliferated in response to Wnt3a truly maintained HSC activity, we carried out a transplantation analysis. Single HSCs were plated in Terasaki plates and treated with Wnt3a or control medium for a period of 6 days. In previous experiments we showed that culturing cells with SLF alone (our control conditions) while inducing proliferation does not induce self-renewal *in vitro*[19]. Each well containing cells that responded to Wnt3a from a single cell was separately injected into lethally irradiated mice, and analysed after 6 weeks of reconstitution (Fig. 3e). If no self-renewal had occurred, only 10% of the mice would be expected to be reconstituted successfully (see Fig. 3 legend). In contrast 100% of the mice contained donor-derived cells (Fig. 3f), suggesting that HSCs had undergone self-renewal in response to purified Wnt3a.

We have established methods to purify significant quantities of pure and active Wnt proteins, which can be used for self-renewal of HSCs and potentially other stem cells. We found that Wnt proteins are unexpectedly hydrophobic and are post-translationally modified by palmitoylation, a property that explains the poor solubility of the proteins. It is interesting to note that the protein products of the *Drosophila porcupine* and *C. elegans mom-1* genes[20,21] have homology with acyl transferases and may catalyse Wnt acylation[22]. Moreover, the Porcupine protein can bind to a domain in Wingless encompassing the acylation site[23]. *porcupine* and *mom-1* have phenotypes similar to *Wnt* alleles and are required in Wnt-producing cells, indicating that the lipid is an integral part of signalling activity. However, overexpression of Wingless in the *Drosophila* embryo can overcome the absence of *porcupine*[24], just as high expression of Wnt3a(C77A) can lead to a modest increase in β-catenin (Fig. 2d). This suggests that the lipid functions to increase the local concentration of Wnt on membranes, and that its absence can be overcome by high levels of expression. Although palmitoylation of secreted proteins seems unusual, there is an intriguing

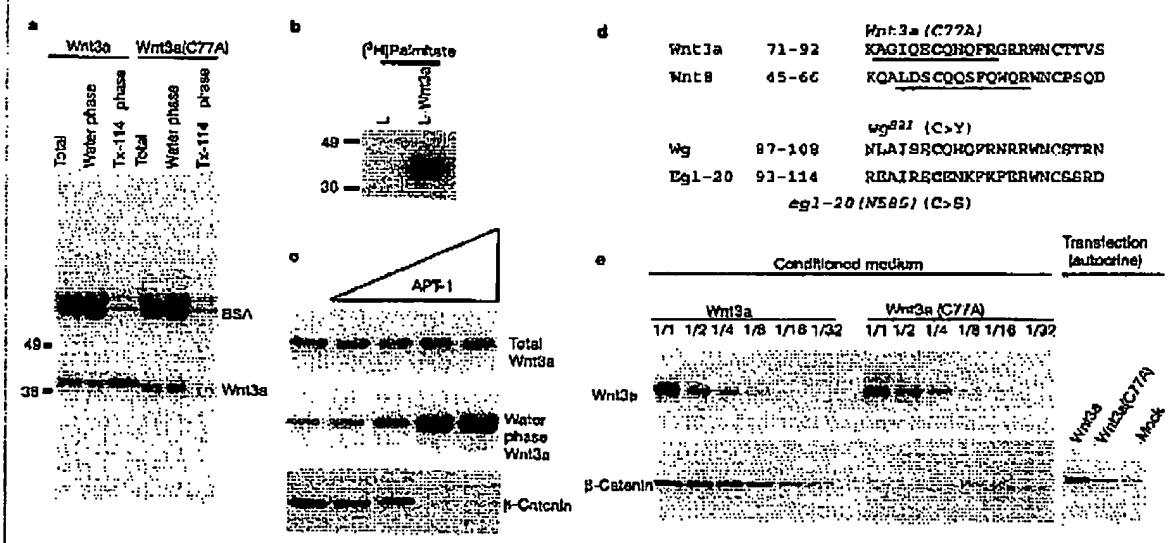
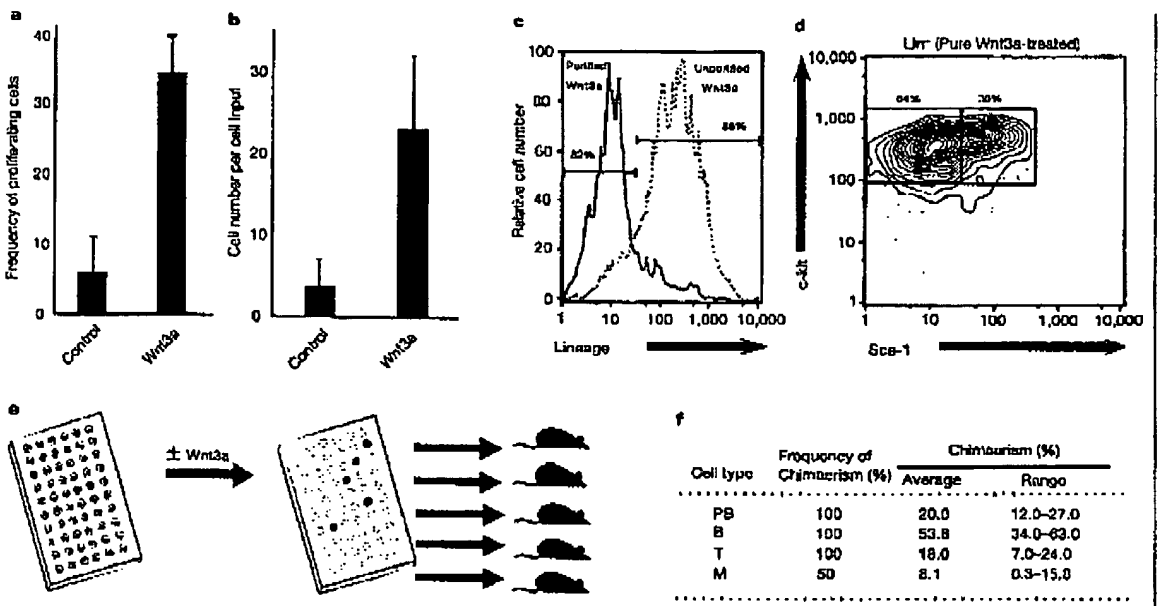


Figure 2 Wnt proteins are palmitoylated on an essential cysteine. a, Triton X-114 phase separation (western blot). Most wild-type Wnt3a separates to the Triton X-114 phase, indicating that it is hydrophobic, but the Wnt3a(C77A) mutant (see d) partitions mostly to the water phase. BSA from serum partitions to the water phase and serves as an internal control. b, *In vivo* labelling of Wnt3a protein with tritiated palmitate. Wnt3a was partially purified from conditioned medium of cells labelled with tritiated palmitate for 5 h. c, APT-1 treatment of Wnt3a (western blot). Treatment of Wnt3a with increasing amounts of APT-1 shifts the Wnt3a protein from the Triton X-114 phase (data not shown) to the water phase (middle panel) and abolishes its activity in the β-catenin stabilization assay. d, Mass spectrometry maps the palmitate modification to a cysteine (bold) in Wnt3a (C77) and in *Drosophila* Wnt8 (C51). Underlined sequence corresponds to the peptide identified in the spectra as being modified. The cysteine is conserved in all known Wnt proteins. A site-directed mutant (Wnt3a(C77A)) was made and used in a and e. The *Drosophila wg*^{S21} (ref. 16) allele has a mutation converting the cysteine into a tyrosine and the *egl-20(N585)* allele in *C. elegans* has a serine instead of the cysteine[17]. These are both loss-of-function alleles. e, The Wnt3a(C77A) mutant protein is secreted from 293 cells at levels similar to wild type, but is not active in increasing β-catenin in target L cells over a range of concentrations tested (western blot). However, the 293 cells transfected with the Wnt3a(C77A) expression construct show a modest increase in β-catenin, indicating that high levels of the mutant can activate Wnt signalling. The Wnt3a(C77A) and wild-type transfected cells express equal levels of Wnt protein.



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Cell type	Frequency of Chimerism (%)	Chimaerism (%) Average	Range
PB	100	20.0	12.0–27.0
B	100	53.8	34.0–63.0
T	100	18.0	7.0–24.0
M	50	8.1	0.3–15.0

Figure 3 HSCs maintain self-renewing fate with reduced differentiation in response to purified Wnt3a. Purified mouse bone marrow (BM) HSCs (c-Kit$^+$, Sca-1$^+$, Thy-1.1^{lo}, Lin$^-$) from Bcl2 transgenic mice[16] were sorted by FACS and plated as single cells into 60-well Terasaki plates. Cells were incubated in X-vivo 5 (Bio Whittaker) containing either purified Wnt3a (about 100 ng ml^{-1}) plus limiting amounts of SLF (7.5 ng ml^{-1}) or SLF (7.5 ng ml^{-1}) alone, as a control. a, Cell growth was monitored over 7 days in culture, and shown as the frequency of responding cells. b, Total cell growth. Cells responded to Wnt3a by proliferating >100-fold (from 1 cell to at least 100 cells) and the total number of cells generated was about greater in the presence of Wnt3a than control conditions. Results are representative of four independent experiments. c, To determine phenotypic characteristics, cells were plated in 96-well plates and incubated in the presence of purified or unpurified Wnt3a. After 7 days in culture, most cells treated with purified Wnt3a (at 100 ng ml^{-1}) were negative for lineage markers (solid line) whereas most treated with unpurified Wnt3a (200 ng ml^{-1} in the medium; Table 1) strongly upregulated lineage markers (dotted line). d, FACS analysis of purified Wnt3a-treated cells. The lineage-negative population is distributed into c-Kit$^+$ and Sca-1$^+$ HSCs and c-Kit$^+$ and Sca-1$^-$ myeloid progenitors. e, Purified mouse BM HSCs were plated singly into 60-well Terasaki plates and treated with Wnt3a for 6 days, then all cells generated from the single cell were transplanted individually into lethally irradiated mice along with 300,000 rescuing BM cells. f, Peripheral blood (PB) from each transplanted mouse was analysed after 6 weeks for reconstitution along both lymphoid (B and T) and myeloid (M) lineages. On the basis of the reconstitution efficiency of single transplanted HSCs, 1 of 10 (10%) resting HSCs and probably 1 of 50 (2%) cycling HSCs reconstitute. A 50% reconstitution rate suggests at least a fivefold and probably a 15–25-fold expansion in HSCs per transplant. Fivefold expansion is probably an underestimate as HSCs transplanted in low numbers lead to low and variable reconstitution. But our finding that Wnt3a-treated HSCs on transplantation lead to an average chimaerism of 20% (range 12–27%) in the context of a competitive reconstitution suggests a greater than fivefold expansion of functional HSCs.

parallel between Wnt and hedgehog signalling, as the hedgehog protein is also palmitoylated[25]. □

Methods

Purification of Wnt3a

Mouse L cells (American Type Culture Collection (ATCC) CRL-2648) were cultured in DMEM medium, 10% fetal bovine serum (FBS) and antibiotics. These cells were stably transfected with a vector containing the Wnt3a complementary DNA under the control of the PGK promoter, and G418-resistant clones were selected and screened for production of Wnt3a protein (ATCC CRL-2647). *Drosophila* S2 cells were used to produce the *Drosophila* Wnt8 protein, which was expressed from a heat-shock promoter[26]. Two litres of 0.2-μm filtered medium from L-Wnt3a cells, conditioned for 4 days, was adjusted to 1% Triton X-100, filtered and applied to blue (Cibacron blue) Sepharose HP (Amersham Biosciences) column (bed volume of 120 ml), which was previously equilibrated in binding buffer (150 mM KCl, 20 mM Tris-HCl, 1% CHAPS, pH 7.5). The column was then washed with four column volumes of binding buffer. Bound proteins were eluted with a single step to 1.5 M KCl, 20 mM Tris-HCl, 1% CHAPS, pH 7.5. Wnt3a eluted in two pools, each of which contained similar amounts of Wnt3a protein; however, the second pool contained significantly less total protein than the first (30.6 mg total protein in the first pool and 2.10 mg in the second pool). Fractions from this second pool were combined, concentrated to 12.5 ml on a Centricon 30 ultrafiltration device (Amicon), and fractionated on a HiLoad 26/60 Superdex 200 column (Amersham Biosciences) in phosphate buffered saline (PBS), 1% CHAPS, pH 7.5. Fractions containing Wnt3a were then fractionated on a 1-ml HiTrap Heparin column (Amersham Biosciences) in a single step elution from PBS, 1% CHAPS to PBS, 1% CHAPS, 1 M NaCl. N-terminal sequence of 1 μg purified Wnt3a was obtained by automated Edman degradation on a Procise 494 A/H sequencer. Isolated Wnt3a begins with residue 19 of the predicted amino acid sequence (SYPIWWSLAVGPQYS), indicating that the protein is proteolytically processed to remove the signal sequence. For a detailed protocol, see http://www.stanford.edu/~anusse/wntwindow.html.

Triton X-114 phase separation

Wnt3a-conditioned medium was mixed 1:1 with ice cold 4.5% Triton X-114, 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, incubated on ice for 5 min, then at 31 °C for 5 min, and centrifuged at 2,000*g* at 31 °C for 5 min. The top, aqueous phase was separated from the bottom Triton X-114 phase and equal volumes were immunoblotted with the anti-Wnt3a antibody.

In vivo labelling of Wnt3a with palmitate

L and L-Wnt3a cells were cultured in 10-cm plates for 3 days after a 1:10 split. [9,10(n)-3H] palmitic acid (Amersham Biosciences) was added to the medium at a concentration of 0.1 mCi ml^{-1} and incubated for 5 h at 37 °C. The medium was filtered, CHAPS was added to a concentration of 1%, and then re-filtered. The individual medium was fractionated on 1-ml HiTrap blue Sepharose columns (Amersham Biosciences) as described above. The Wnt3a-containing fractions or analogous fractions were precipitated with trichloroacetic acid and analyzed by gel-electrophoresis and autoradiography.

Liquid chromatography tandem mass spectrometry

Purified Wnt3a and *Drosophila* Wnt8 were precipitated with trichloroacetic acid, re-suspended, alkylated and reduced as described[27]. The sample was split into three aliquots, digested separately with trypsin, subtilisin and elastase, and the resulting peptide mixtures

were recombined and analysed by MudPIT as described[28] with modifications as described[27] on a Finnigan LCQ-Deca. Tandem mass spectra were searched against a database of predicted open reading frames to which common contaminants such as keratin and trypsin were added. Search results were filtered and grouped using the DTASelect program[29] and identifications confirmed through manual evaluation of spectra. The data were subsequently searched with a differential modification on cysteine of 238 to identify sites of palmitoylation. We also observed this peptide in its unpalmitoylated form, and at present we cannot distinguish whether the lipid is labile and lost during the manipulation of the sample or whether there is a pool of unmodified Wnt3a present in the preparation. We found the following masses [(M + H) +]: Wnt3a peptide unmodified: 1374.51 (predicted, 1374.465); Wnt3a peptide modified: 1556.10 (predicted, 1555.465); *Drosophila* Wnt8 peptide unmodified: 1583.37 (predicted, 1583.667); *Drosophila* Wnt8 peptide modified: 1764.23 (predicted, 1764.667). Although the tandem mass spectrometry analysis of Wnt3a and *Drosophila* Wnt8 identified 65% and 90% of the primary amino acid sequences, respectively, we did not obtain evidence for additional lipid modifications on other residues (S, T, Y, K, R).

Acyl-protein thioesterase treatment of Wnt3a

A total of 100 ng Wnt3a was treated in the presence of 1 µg BSA with 1, 10, 100 or 1,000 ng APT-1 (provided by A. Gilman) in buffer (PBS, 1% CHAPS, 1 M NaCl) in a total volume of 10 µl and incubated overnight at 30 °C. The reaction products were analysed in the β-catenin stabilization assay on L cells and in the Triton X-114 phase separation assay.

HSC isolation and assays

HSCs were sorted from mouse bone marrow of bcl2 transgenic mice using antibodies as described[30]. Cells were sorted on expression of c-Kit, Sca-1, low levels of Thy-1.1 and low to negative levels of lineage markers (Lin) using clonecyte software and the single cell deposition unit (Becton Dickinson). See Supplementary Information.

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Deficiency of the adaptor SLP-65 in pre-B-cell acute lymphoblastic leukaemia

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Acute lymphoblastic leukaemia (ALL) is the commonest form of childhood malignancy, and most cases arise from B-cell clones arrested at the pre-B-cell stage of differentiation[1,2]. The molecular events that arrest pre-B-cell differentiation in the leukaemic pre-B cells have not been well characterized. Here we show that the differentiation regulator SLP-65 (an adaptor protein also called BLNK or BASH[3–6]) inhibits pre-B-cell leukaemia in mice. Reconstitution of SLP-65 expression in a SLP-65$^{-/-}$ pre-B-cell line led to enhanced differentiation *in vitro* and prevented the development of pre-B-cell leukaemia in immune-deficient mice. Tyrosine 96 of SLP-65 was required for this activity. The murine SLP-65$^{-/-}$ pre-B-cell leukaemia resembles human childhood pre-B ALL. Indeed, 16 of the 34 childhood pre-B ALL samples that were tested showed a complete loss or drastic reduction of SLP-65 expression. This loss is probably due to the incorporation of alternative exons into SLP-65 transcripts, leading to premature

